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**(54) Title:** METHOD OF TREATING TUMOR CELLS BY INHIBITING GROWTH FACTOR RECEPTOR FUNCTION

**(57) Abstract**

A method of inhibiting growth of tumor cells which overexpress a growth factor receptor or growth factor by treatment of the cells with antibodies which inhibit the growth factor receptor function is disclosed. A method of treating tumor cells with antibodies which inhibit growth factor receptor function, and with cytotoxic factor(s) such as tumor necrosis factor, is also disclosed. By inhibiting growth factor receptor functions tumor cells are rendered more susceptible to cytotoxic factors.

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METHOD OF TREATING TUMOR CELLS  
BY INHIBITING GROWTH FACTOR RECEPTOR FUNCTION

Field of the Invention

5        This invention is in the fields of immunology and cancer  
diagnosis and therapy. More particularly it concerns antibodies  
specifically binding growth factor receptors, hybridomas that  
produce these antibodies, immunochemicals made from the antibodies,  
and diagnostic methods that use the antibodies. The invention also  
10       relates to the use of the antibodies alone or in combination with  
cytotoxic factor(s) in therapeutic methods. Also encompassed by  
the invention is an assay for tyrosine kinases that are involved in  
tumorigenesis.

15       Background of the Invention

Macrophages are one of the effector cell types that play an  
important role in immunosurveillance against neoplastic growth in  
vivo. In vitro, cell-mediated cytotoxicity requires selective  
binding between activated macrophages and target cells as well as  
20       the concomitant release of cytotoxic factors. Some of the  
cytotoxic factors secreted by activated macrophages include  
reactive oxygen species such as the superoxide anion and hydrogen  
peroxide, arginase, interleukin 1, and tumor necrosis factor- $\alpha$   
(TNF- $\alpha$ ). Acquired resistance to the toxic effects of these factors  
25       by tumor cells could be one mechanism which leads to the onset and  
spread of tumor formation in vivo.

The observation that TNF- $\alpha$  can act as a potent effector of the  
macrophage-mediated antitumor response provides a rationale for its  
30       use in further studies on the regulation of tumorigenesis in vivo  
and tumor cell growth in vitro. The genes encoding TNF- $\alpha$  and TNF- $\beta$ ,  
a structurally related cytotoxic protein formerly known as  
lymphotoxin, have been cloned and the corresponding proteins  
expressed in *Escherichia coli*. These proteins display an array of  
35       biological activities, including induction of hemorrhagic necrosis

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of Meth A sarcomas in vivo, inhibition of the growth of certain tumor cells in vitro, synergistic enhancement of the in vitro anticellular effects of IFN- $\gamma$ , activation of human polymorphonuclear neutrophil functions, and inhibition of lipid biosynthesis. Recently, rHuTNF- $\alpha$  was shown to augment the growth of normal diploid fibroblasts in vitro. The divergent proliferative responses in the presence of rHuTNF- $\alpha$  are sometimes related to variations in TNF binding.

Growth factors and their receptors are involved in the regulation of cell proliferation and they also appear to play a key role in oncogenesis. Of the known proto-oncogenes, three are related to a growth factor or a growth factor receptor. These genes include *c-sis*, which is homologous to the transforming gene of the simian sarcoma virus and is the B chain of platelet-derived growth factor (PDGF); *c-fms*, which is homologous to the transforming gene of the feline sarcoma virus and is closely related to the macrophage colony-stimulating factor receptor (CSF-1R); an *c-erbB*, which encodes the EGF receptor (EGFR) and is homologous to the transforming gene of the avian erythroblastosis virus (*v-erbB*). The two receptor-related proto-oncogenes, *c-fms* and *c-erbB*, are members of the tyrosine-specific protein kinase family to which many proto-oncogenes belong.

Recently, a novel transforming gene was identified as a result of transfection studies with DNA from chemically induced rat neuroblastomas. This gene, called *neu*, was shown to be related to, but distinct from, the *c-erbB* proto-oncogene. By means of *v-erbB* and human EGFR as probes to screen human genomic and complementary DNA (cDNA) libraries, two other groups independently isolated human *erbB*-related genes that they called HER2 and *c-erbB-2* respectively. Subsequent sequence analysis and chromosomal mapping studies revealed that *c-erbB-2*, and HER2 are species variants of *neu*. A fourth group, also using *v-erbB* as a probe, identified the same

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gene in a mammary carcinoma cell line, MAC 117, where it was found to be amplified five- to ten-fold.

5 This gene, which will be referred to herein as HER2, encodes a new member of the tyrosine kinase family; and is closely related to, but distinct from, the EGFR gene as reported by Coussens et al., Science 230, 1132 (1985). HER2 differs from EGFR in that it is found on band q21 of chromosome 17, as compared to band p11-p13 of chromosome 7, where the EGFR gene is located. Also, the HER2  
10 gene generates a messenger RNA (mRNA) of 4.8 kb, which differs from the 5.8- and 10-kb transcripts for the EGFR gene. Finally, the protein encoded by the HER2 gene is 185,000 daltons, as compared to the 170,000-dalton protein encoded by the EGFR gene. Conversely, on the basis of sequence data, HER2 is more closely related to the  
15 EGFR gene than to other members of the tyrosine kinase family. Like the EGFR protein, the HER2 protein (p185) has an extracellular domain, a transmembrane domain that includes two cysteine-rich repeat clusters, and an intracellular kinase domain, indicating that it is likely to be a cellular receptor for an as yet  
20 unidentified ligand. HER2 p185 is referred to as p185 or the HER2 receptor herein.

Southern analysis of primary human tumors and established tumor-derived cell lines revealed amplification and in some cases  
25 rearrangement of the EGF receptor gene. Amplification was particularly apparent in squamous carcinomas and glioblastomas. The HER2 gene was also found to be amplified in a human salivary gland adenocarcinoma, a renal adenocarcinoma, a mammary gland carcinoma, and a gastric cancer cell line. Recently, Slamon et al., Science 235, 177 (1987) demonstrated that about 30% of primary  
30 human breast carcinoma tumors contained an amplified HER2 gene. Although a few sequence rearrangements were detected, in most tumors there were no obvious differences between amplified and normal HER2 genes. Furthermore, amplification of the HER2 gene

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correlated significantly with the negative prognosis of the disease and the probability of relapse.

5 To investigate the significance of the correlation between over-expression and cellular transformation as it has been observed for proto-oncogenes *c-mos* and *N-myc*, a HER2 expression vector and a selection scheme that permitted sequence amplification after transfection of mouse NIH 3T3 cells was employed by Hudziak et al., Proc. Natl. Acad. Sci. (USA) 84, 7159 (1987). Amplification of the  
10 unaltered HER2 gene in NIH 3T3 cells lead to over-expression of p185 as well as cellular transformation and tumor formation in athymic mice.

15 The effects of antibodies specifically binding growth factors or growth factor receptors has been studied. Examples are discussed below.

Rosenthal et al., Cell 46, 301 (1986) introduced a human TGF- $\alpha$  cDNA expression vector into established non-transformed rat  
20 fibroblast cells. Synthesis and secretion of TGF- $\alpha$  by these cells resulted in loss of anchored-dependent growth and induced tumor formation in nude mice. Anti-human TGF- $\alpha$  monoclonal antibodies prevented the rat cells from forming colonies in soft agar, i.e. loss of anchorage dependence. Gill et al., in J. Biol. Chem. 259  
25 7755 (1984) disclose monoclonal antibodies specific for EGF receptor which were inhibitors of EGF binding and antagonists of EGF-stimulated tyrosine protein kinase activity.

30 Drebin et al., in Cell 41 695 (1985) demonstrated that exposure of a neu-oncogene-transformed NIH 3T3 cell to monoclonal antibodies reactive with the *neu* gene product, cause the *neu*-transformed NIH 3T3 cell to revert to a non-transformed phenotype as determined by anchorage independent growth. Drebin et al., in Proc. Natl. Acad. Sci. 83, 9129 (1986) demonstrated that in vivo treatment with a  
35 monoclonal antibody (IgG2a isotype) specifically binding the

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protein encoded by the *neu* oncogene significantly inhibited the tumorigenic growth of *neu*-transformed NIH 3T3 cells implanted into nude mice.

5        Akiyama *et al.* in Science 232, 1644 (1986) raised antibodies against a synthetic peptide corresponding to 14 amino acid residues at the carboxy-terminus of the protein deduced from the *c-erbB-2* (HER2) nucleotide sequence.

10        Growth factors have been reported to interact in both a synergistic and an antagonistic manner. For example, TGF- $\alpha$  and TGF- $\beta$  synergistically enhance the growth of NRK-49F fibroblasts, whereas PDGF down regulates EGF receptor function on 3T3 cells. A  
15        variety of transformed cells secrete factors which are believed to stimulate growth by an autocrine mechanism. Sugarman *et al.*, Cancer Res. 47, 780 (1987) demonstrated that under certain conditions, growth factors can block the antiproliferative effects of TNF- $\alpha$  on sensitive tumor cells. Specifically, epidermal growth factor (EGF) and recombinant human transforming growth factor- $\alpha$   
20        (rHuTGF- $\alpha$ ) were shown to interfere with the in vitro antiproliferative effects of recombinant human tumor necrosis factor- $\alpha$  (rHuTNF- $\alpha$ ) and - $\beta$  on a human cervical carcinoma cell line, ME-180. The inhibitory effect could be observed at EGF or rHuTGF- $\alpha$  concentrations of 0.1 to 100 ng/ml, and was maximal between 1 and  
25        10 ng/ml. This response was apparently not due to down regulation of the TNF receptor or to alteration of the affinity of TNF- $\alpha$  for its receptor. Since the antiproliferative effect of recombinant human interferon- $\gamma$  was not significantly affected by the presence of EGF or rHuTGF- $\alpha$ , the inhibition was specific for recombinant  
30        TNFs and was not due solely to enhanced proliferation induced by the growth factors. Neither growth factor had a substantial protective effect on the synergistic cytotoxicity observed when tumor cells were exposed simultaneously to rHuTNF- $\alpha$  and recombinant human interferon- $\gamma$ . TGF- $\beta$  can also interfere with the  
35        antiproliferative effects of rHuTNF- $\alpha$  in vitro. At concentrations

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of less than 1 ng/ml, TGF- $\beta$  significantly antagonized the cytotoxic effects of rHuTNF- $\alpha$  on NIH 3T3 fibroblasts. Since EGF, platelet-derived growth factor, and TGF- $\beta$  all enhanced NIH 3T3 cell proliferation, but only TGF- $\beta$  interfered with rHuTNF- $\alpha$  cytotoxicity, the protective effects of TGF- $\beta$  were not related in a simple manner to enhanced cell proliferation. rHuTGF- $\alpha$  and TGF- $\beta$  did not have a significant protective effect against rHuTNF- $\alpha$ -mediated cytotoxicity on two other tumor cell lines, BT-20 and L-929 cells.

It is an object of the subject invention to provide antibodies capable of inhibiting growth factor receptor function.

It is a further object of the invention to provide an improved assay for the HER2 receptor.

It is a further object of the invention to provide improved methods of tumor therapy.

It is a further object of the invention to provide a method of inhibiting the growth of tumor cells which overexpress a growth factor receptor and/or growth factor.

It is a further object of the invention to provide a method for treating a tumor by treatment of the tumor cells with antibodies capable of inhibiting growth factor receptor function, and with cytotoxic factors such as tumor necrosis factor.

A still further object of the invention is to provide an assay for tyrosine kinases that may have a role in tumorigenesis.

Other objects, features and characteristics of the present invention will become apparent upon consideration of the following description and the appended claims.



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Summary of the Invention

The subject invention relates to monoclonal antibodies specifically binding the external domain of the HER2 receptor. The invention also relates to an assay for the HER2 receptor comprising exposing cells to antibodies specifically binding the extracellular domain of the HER2 receptor, and determining the extent of binding of said antibodies to said cells. Another embodiment of the invention relates to a method of inhibiting growth of tumor cells by administering to a patient a therapeutically effective amount of antibodies capable of inhibiting the HER2 receptor function. A further embodiment of the invention relates to administering a therapeutically effective amount of antibodies capable of inhibiting growth factor receptor function, and a therapeutically effective amount of a cytotoxic factor. A still further embodiment of the invention is an assay for tyrosine kinases that may have a role in tumorigenesis comprising exposing cells suspected to be TNF- $\alpha$  resistant to TNF- $\alpha$ , isolating those cell which are TNF- $\alpha$  resistant, screening the isolated cells for increased tyrosine kinase activity, and isolating receptors and other proteins having increased tyrosine kinase activity.

Brief Description of the Drawings

Figure 1a shows TNF- $\alpha$  resistance of NIH 3T3 cells expressing various levels of HER2 p185. Figure 1b shows macrophage cytotoxicity assays for NIH 3T3 cells expressing various levels of HER2 p185.

Figure 2 demonstrates the level of TNF- $\alpha$  binding for a control cell line (NIH 3T3 neo/dhfr) and for a cell line overexpressing HER2 p185 (HER2-3800).

Figure 3 shows inhibition of SK BR3 cell growth by anti-HER2 monoclonal antibodies.

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Figure 4 is a dose response curve comparing the effect of an irrelevant monoclonal antibody (anti-HBV) and the effect of monoclonal antibody 4D5 (anti-HER2) on the growth of SK BR3 cells in serum.

5

Figures 5a, 5b and 6a show percent viability of SK BR3 cells as a function of increasing TNF- $\alpha$  concentration and anti-HER2 p185 monoclonal antibody concentration. Each Figure shows the results for a different anti-HER2 p185 monoclonal antibody. Figure 6b is a control using an irrelevant monoclonal antibody.

10

Figure 7 shows percent viability of MDA-MB-175-VII cells as a function of increasing TNF- $\alpha$  concentration and anti-HER2 p185 monoclonal antibody concentration.

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Figure 8 shows percent viability of NIH 3T3 cells overexpressing HER2 p185 as a function of increasing TNF- $\alpha$  concentration and anti-HER2 p185 monoclonal antibody concentration.

20

#### Detailed Description of the Invention

A new application of antibodies to inhibit the growth of tumor cells has been discovered. Surprisingly, it has been found that by inhibiting growth factor receptor function, e.g. the HER2 receptor function, cell growth is inhibited, and the cells are rendered more susceptible to cytotoxic factors. Thus, for example, breast cancer cells which are refractory to TNF- $\alpha$  alone can be made susceptible to TNF- $\alpha$  if the cells are first treated with antibodies which inhibit growth factor receptor function. The increase of susceptibility has been demonstrated using the HER2 receptor and monoclonal antibodies directed against the HER2 receptor, and tumor necrosis factor- $\alpha$ .

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The method of this invention is useful in the therapy of malignant or benign tumors of mammals where the abnormal growth rate of the tumor is dependent upon growth factor receptors.

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Abnormal growth rate is a rate of growth which is in excess of that required for normal homeostasis and is in excess of that for normal tissues of the same origin. Many of these tumors are dependent upon extracellular sources of the growth factor recognized by the receptor, or upon synthesis of the growth factor by the tumor cell itself. This latter phenomenon is termed "autocrine" growth.

The methods of the subject invention is applicable where the following conditions are met:

- (1) the growth factor receptor and/or ligand (growth factor) is expressed, and tumor cell growth depends upon the growth factor receptor biological function;
- (2) antibodies specifically binding the growth factor receptor and/or ligand inhibit the growth factor receptor biological function.

While not wishing to be constrained to any particular theory of operation of the invention, it is believed that the antibodies inhibit growth factor receptor biological function in one or more of the following ways:

- (a) The antibodies bind to the extracellular domain of the receptor and inhibit the ligand from binding the receptor;
- (b) The antibodies bind the ligand (the growth factor) itself and inhibit the ligand from binding the receptor;
- (c) The antibodies down regulate the growth factor receptor;
- (d) The antibodies sensitize tumor cells to the cytotoxic effects of a cytotoxic factor such as  $\text{TNF-}\alpha$ ;
- (e) The antibodies inhibit the tyrosine kinase activity of the receptor.

In cases (f) and (g), the antibodies inhibit growth factor receptor biological function indirectly by mediating cytotoxicity via a targeting function:

- (f) The antibodies belong to a sub-class or isotype that upon complexing with the receptor activates serum complement and/or

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mediate antibody-dependent cellular cytotoxicity (ADCC), e.g. IgG2a antibodies;

(g) The antibodies which bind the receptor or growth factor are conjugated to a toxin (immunotoxins);

5 Advantageously antibodies are selected which greatly inhibit the receptor function by binding the steric vicinity of the ligand binding site of the receptor (blocking the receptor), and/or which bind the growth factor in such a way as to prevent (block) the ligand from binding to the receptor. These antibodies are selected  
10 using conventional in vitro assays for selecting antibodies which neutralize receptor function. Antibodies that act as ligand agonists by mimicking the ligand are discarded by conducting suitable assays as will be apparent to those skilled in the art. For certain tumor cells, the antibodies inhibit an autocrine growth  
15 cycle (i.e. where a cell secretes a growth factor which then binds to a receptor of the same cell). Since some ligands, e.g. TGF- $\alpha$ , are found lodged in cell membranes, the antibodies serving a targeting function are directed against the ligand and/or the receptor.

20  
Certain tumor cells secrete growth factors that are required for normal cellular growth and division. These growth factors, however, can under some conditions stimulate unregulated growth of the tumor cell itself, as well as adjacent non-tumor cells, and can  
25 cause a tumor to form.

Epidermal Growth Factor (EGF) has dramatic stimulatory effects on cell growth. In purified receptor preparations, the EGF receptor is a protein kinase that is activated by the binding of  
30 EGF. Substrate proteins for this kinase are phosphorylated on tyrosine residues. The receptors for insulin, platelet-derived growth factor (PDGF) and other growth hormones also are tyrosine-specific kinases. It is believed that ligand binding to the receptor triggers phosphorylation of certain proteins by the  
35 receptor and in this way stimulates cell growth. About one-third

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of the known oncogenes encode proteins that phosphorylate tyrosine residues on other proteins. It is believed that these oncogene products trigger responses analogous to the responses of cells to growth factors and hormones. The *erbB* oncogene product is a portion of the EGF receptor that lacks the hormone-binding domain and may give rise to a constitutive growth-stimulating signal.

One embodiment of this invention is a method of inhibiting the growth of tumor cells by administering to a patient a therapeutically effective amount of antibodies that inhibit the HER2 receptor biological function of tumor cells.

Overexpression of growth factor receptors increases the resistance of cells to TNF as demonstrated below. Overexpression of the HER1 receptor (EGF receptor), met receptor-like protooncogene product, and HER2 receptor all show this increased resistance. It is shown in the Examples below that amplified expression of HER2, which encodes the HER2 receptor (p185), induces resistance of NIH 3T3 cells to the cytotoxic effects of macrophages or TNF- $\alpha$ . Induction of NIH 3T3 cell resistance to TNF- $\alpha$  by overexpression of p185 is accompanied by alterations in the binding of TNF- $\alpha$  to its receptor. Overexpression of p185 is also associated with resistance of certain human breast tumor cell lines to the cytotoxic effects of TNF- $\alpha$ .

In another embodiment of the invention, tumor cells are treated by (1) administering to a patient antibodies directed against the growth factor and/or its receptor, that inhibit the biological function of the receptor and that sensitize the cells to cytotoxic factors such as TNF, and (2) administering to the patient cytotoxic factor(s) or other biological response modifiers which activate immune system cells directly or indirectly to produce cytotoxic factors.

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The cytotoxic factor, such as TNF- $\alpha$ , exerts its cytostatic (cell growth suppressive) and cytotoxic (cell destructive) effect. Examples of useful cytotoxic factors are TNF- $\alpha$ , TNF- $\beta$ , IL-1, IFN- $\gamma$  and IL-2, and chemotherapeutic drugs such as 5FU, vinblastine, actinomycin D, etoposide, cisplatin, methotrexate, and doxorubicin. Cytotoxic factors can be administered alone or in combination. In a still further embodiment of the invention, the patient is treated with antibodies which inhibit receptor function, and with autologous transfer therapy, e.g. LAK or TIL cells.

10

Tumor necrosis factors are polypeptides produced by mitogen-stimulated macrophages or lymphocytes which are cytotoxic for certain malignantly transformed cells. The anti-tumor effect of TNF- $\alpha$  is known to be synergistically potentiated by interferons. The anti-tumor effect of TNF- $\alpha$  and TNF- $\beta$  in admixture are additive, as are the antiviral effects of interferons alpha and beta.

15

The tumor necrosis factors include TNF- $\alpha$  and TNF- $\beta$ . The former is described together with methods for its synthesis in recombinant cell culture, in U.S. Patent 4,650,674, and in European Patent Application 0168214; the latter is described in European Patent Application 0164965. The TNF- $\alpha$  and TNF- $\beta$  described in these patent documents includes cytotoxic amino acid sequence and glycosylation variants. TNF- $\alpha$  and TNF- $\beta$  from non-recombinant sources are also useful in the method of this invention.

20

25

The preferred TNF is mature human TNF- $\alpha$  from recombinant microbial cell culture. The TNF ordinarily will have a cytolytic activity on susceptible L-M murine cells of greater than about  $1 \times 10^6$  units/mg, wherein a unit is defined as set forth in the above-described patent application.

30

In another embodiment of the subject invention, one or more additional cytokines and/or cytotoxic factors are administered with TNF- $\alpha$ , egs. interferons, interleukins, and chemotherapeutic drugs.

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5 The compositions herein include a pharmaceutically acceptable vehicle such as those heretofore used in the therapeutic administration of interferons or TNF, e.g. physiological saline or 5% dextrose, together with conventional stabilizers and/or excipients such as human serum albumin or mannitol. The compositions are provided lyophilized or in the form of sterile aqueous solutions.

10 Several variables will be taken into account by the ordinary artisan in determining the concentration of TNF in the therapeutic compositions and the dosages to be administered. Therapeutic variables also include the administration route, and the clinical condition of the patient.

15 The cytotoxic factor(s) and antibodies inhibiting growth factor receptor function are administered together or separately. If the latter, advantageously the antibodies are administered first and the TNF thereafter within 24 hours. It is within the scope of this invention to administer the TNF and antibodies in multiple cycles, depending upon the clinical response of the patient. The TNF and antibodies are administered by the same or separate routes, for example by intravenous, intranasal or intramuscular administration.

25 The method of the subject invention can be used with tumor cells which overexpress growth factor receptor and/or ligand where antibodies can be produced which inhibit the growth factor receptor function. A cell (e.g. breast tumor cell) overexpresses a growth factor receptor if the number of receptors on the cell exceeds the number on the normal healthy cell (e.g. normal breast tissue cell). Examples of carcinomas where the HER2 receptor is overexpressed (and thus the method of the subject invention is applicable), are human breast, renal, gastric and salivary gland carcinomas.

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A further embodiment of the invention is an assay for identifying receptors and other proteins having increased tyrosine kinase activity, and for identifying oncogenes that transform cells. Amplification of certain oncogenes encoding tyrosine kinases correlates with TNF- $\alpha$  resistance. If cells are selected for resistance to TNF- $\alpha$ , some of these will have increased tyrosine kinase activity. Some of the tyrosine kinases will be receptors. The genes encoding the tyrosine kinases are then cloned using standard techniques for the cloning of genes. Identification of the receptor or other protein permits the design of reagents which inhibit receptor (or other protein) function and induce cellular sensitivity to cytotoxic factors as demonstrated herein with HER2. Identification of the receptor also permits subsequent identification of the receptor's ligand. The assay comprises exposing cells suspected to be TNF- $\alpha$  sensitive to TNF- $\alpha$ , and isolating those cells which are TNF- $\alpha$  resistant. The TNF- $\alpha$  resistant cells are then screened for increased tyrosine kinase activity, and receptors and other proteins having increased tyrosine kinase activity are isolated.

#### Antibodies

In accordance with this invention, monoclonal antibodies specifically binding growth factors or growth factor receptors such as the HER2 receptor, were isolated from continuous hybrid cell lines formed by the fusion of antigen-primed immune lymphocytes with myeloma cells. Advantageously, the monoclonal antibodies of the subject invention which bind growth factor receptors, bind the extracellular domain of the receptors. In another embodiment of the invention, polyclonal antibodies specifically binding the growth factors or growth factor receptors are used.

The antibodies of the subject invention which are used in tumor therapy advantageously inhibit tumor cell growth greater than 20%, and most advantageously greater than 50%, in vitro. These antibodies are obtained through screening (see, for example, the



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discussion relating to Figure 3). The anti-HER2 receptor monoclonal antibodies of the subject invention which are used in tumor therapy are capable of inhibiting serum activation of the receptor.

5

Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional antibody (polyclonal) preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. Monoclonal antibodies are useful to improve the selectivity and specificity of diagnostic and analytical assay methods using antigen-antibody binding. A second advantage of monoclonal antibodies is that they are synthesized by the hybridoma culture, uncontaminated by other immunoglobulins. Monoclonal antibodies may be prepared from supernatants of cultured hybridoma cells or from ascites induced by intra-peritoneal inoculation of hybridoma cells into mice.

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The hybridoma technique described originally by Kohler and Milstein, Eur. J. Immunol. 6, 511 (1976) has been widely applied to produce hybrid cell lines that secrete high levels of monoclonal antibodies against many specific antigens.

20

The route and schedule of immunization of the host animal or cultured antibody-producing cells therefrom are generally in keeping with established and conventional techniques for antibody stimulation and production. Applicants have employed mice as the test model although it is contemplated that any mammalian subject including human subjects or antibody producing cells therefrom can be manipulated according to the processes of this invention to serve as the basis for production of mammalian, including human, hybrid cell lines.

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After immunization, immune lymphoid cells are fused with myeloma cells to generate a hybrid cell line which can be cultivated and subcultivated indefinitely, to produce large quantities of monoclonal antibodies. For purposes of this invention, the immune lymphoid cells selected for fusion are lymphocytes and their normal differentiated progeny, taken either from lymph node tissue or spleen tissue from immunized animals. Applicants prefer to employ immune spleen cells, since they offer a more concentrated and convenient source of antibody producing cells with respect to the mouse system. The myeloma cells provide the basis for continuous propagation of the fused hybrid. Myeloma cells are tumor cells derived from plasma cells.

It is possible to fuse cells of one species with another. However, it is preferred that the source of immunized antibody producing cells and myeloma be from the same species.

The hybrid cell lines can be maintained in culture in vitro in cell culture media. The cell lines of this invention can be selected and/or maintained in a composition comprising the continuous cell line in the known hypoxanthine-aminopterin-thymidine (HAT) medium. In fact, once the hybridoma cell line is established, it can be maintained on a variety of nutritionally adequate media. Moreover, the hybrid cell lines can be stored and preserved in any number of conventional ways, including freezing and storage under liquid nitrogen. Frozen cell lines can be revived and cultured indefinitely with resumed synthesis and secretion of monoclonal antibody. The secreted antibody is recovered from tissue culture supernatant by conventional methods such as precipitation, ion exchange chromatography, affinity chromatography, or the like.

While the invention is demonstrated using mouse monoclonal antibodies, the invention is not so limited; in fact, human antibodies may be used and may prove to be preferable. Such

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antibodies can be obtained by using human hybridomas (Cote et al., Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, p. 77 (1985)). In fact, according to the invention, techniques developed for the production of "chimeric antibodies" (Morrison et al. Proc. Natl. Acad. Sci. 81, 6851 (1984); Neuberger et al., Nature 312, 604 (1984); Takeda et al., Nature 314, 452 (1985)) by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity (such as ability to activate human complement and mediate ADCC) can be used; such antibodies are within the scope of this invention.

As another alternative to the cell fusion technique, EBV immortalized B cells are used to produce the monoclonal antibodies of the subject invention. Other methods for producing monoclonal antibodies such as recombinant DNA, are also contemplated.

The immunochemical derivatives of the antibodies of this invention that are of prime importance are (1) immunotoxins (conjugates of the antibody and a cytotoxic moiety) and (2) labeled (e.g. radiolabeled, enzyme-labeled, or fluorochrome-labeled) derivatives in which the label provides a means for identifying immune complexes that include the labeled antibody. The antibodies are also used to induce lysis through the natural complement process, and to interact with antibody dependent cytotoxic cells normally present.

#### Immunotoxins

The cytotoxic moiety of the immunotoxin may be a cytotoxic drug or an enzymatically active toxin of bacterial or plant origin, or an enzymatically active fragment ("A chain") of such a toxin. Enzymatically active toxins and fragments thereof used are diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from Pseudomonas aeruginosa), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, Aleurites

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fordii proteins, dianthin proteins, Phytolacca americana proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, and enomycin. In another embodiment, the antibodies are conjugated to small molecule anticancer drugs. Conjugates of the monoclonal antibody and such cytotoxic moieties are made using a variety of bifunctional protein coupling agents. Examples of such reagents are SPDP, IT, bifunctional derivatives of imidoesters such as dimethyl adipimidate HCl, active esters such as disuccinimidyl suberate, aldehydes such as glutaraldehyde, bis-azido compounds such as bis (p-azidobenzoyl) hexanediamine, bis-diazonium derivatives such as bis-(p-diazoniumbenzoyl)-ethylenediamine, diisocyanates such as tolylene 2,6-diisocyanate, and bis-active fluorine compounds such as 1,5-difluoro-2,4-dinitrobenzene. The lysing portion of a toxin may be joined to the Fab fragment of the antibodies.

Advantageously, monoclonal antibodies specifically binding the external domain of the target growth factor receptor, e.g. HER2 receptor, are conjugated to ricin A chain. Most advantageously the ricin A chain is deglycosylated and produced through recombinant means. An advantageous method of making the ricin immunotoxin is described in Vitetta et al., Science 238, 1098 (1987).

When used to kill human cancer cells in vitro for diagnostic purposes, the conjugates will typically be added to the cell culture medium at a concentration of at least about 10 nM. The formulation and mode of administration for in vitro use are not critical. Aqueous formulations that are compatible with the culture or perfusion medium will normally be used. Cytotoxicity may be read by conventional techniques to determine the presence or degree of cancer.

Cytotoxic radiopharmaceuticals for treating cancer may be made by conjugating radioactive isotopes (e.g. I, Y, Pr) to the

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antibodies. The term "cytotoxic moiety" as used herein is intended to include such isotopes.

5 In another embodiment, liposomes are filled with a cytotoxic drug and the liposomes are coated with antibodies specifically binding a growth factor receptor. Since there are many receptor sites, this method permits delivery of large amounts of drug to the correct cell type.

10 Antibody Dependent Cellular Cytotoxicity

15 The present invention also involves a method based on the use of antibodies which are (a) directed against growth factor receptors such as HER2 p185, and (b) belong to a subclass or isotype that is capable of mediating the lysis of tumor cells to which the antibody molecule binds. More specifically, these antibodies should belong to a subclass or isotype that, upon complexing with growth factor receptors, activates serum complement and/or mediates antibody dependent cellular cytotoxicity (ADCC) by activating effector cells such as natural killer cells or  
20 macrophages.

25 The present invention is also directed to the use of these antibodies, in their native form, for therapy of human tumors. For example, many IgG2a and IgG3 mouse antibodies which bind tumor associated cell surface antigens can be used in vivo for tumor therapy. In fact, since HER2 p185 is present on a variety of tumors, the subject antibodies and their therapeutic use have general applicability.

30 Biological activity of antibodies is known to be determined, to a large extent, by the Fc region of the antibody molecule (Uanane and Benacerraf, Textbook of Immunology, 2nd Edition, Williams & Wilkins, p. 218 (1984)). This includes their ability to activate complement and to mediate antibody-dependent cellular  
35 cytotoxicity (ADCC) as effected by leukocytes. Antibodies of

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different classes and subclasses differ in this respect, and, according to the present invention, antibodies of those classes having the desired biological activity are selected. For example, mouse immunoglobulins of the IgG3 and IgG2a class are capable of activating serum complement upon binding to the target cells which express the cognate antigen.

In general, antibodies of the IgG2a and IgG3 subclass and occasionally IgG1 can mediate ADCC, and antibodies of the IgG3, and IgG2a and IgM subclasses bind and activate serum complement. Complement activation generally requires the binding of at least two IgG molecules in close proximity on the target cell. However, the binding of only one IgM molecule activates serum complement.

The ability of any particular antibody to mediate lysis of the tumor cell target by complement activation and/or ADCC can be assayed. The tumor cells of interest are grown and labeled in vivo; the antibody is added to the tumor cell culture in combination with either serum complement or immune cells which may be activated by the antigen antibody complexes. Cytolysis of the target tumor cells is detected by the release of label from the lysed cells. In fact, antibodies can be screened using the patient's own serum as a source of complement and/or immune cells. The antibody that is capable of activating complement or mediating ADCC in the in vitro test can then be used therapeutically in that particular patient.

Antibodies of virtually any origin can be used according to this embodiment of the present invention provided they bind growth factor receptors such as HER2 p185 and can activate complement or mediate ADCC. Monoclonal antibodies offer the advantage of a continuous, ample supply. In fact, by immunizing mice with, for example, HER2 p185, establishing hybridomas making antibodies to p185 and selecting hybridomas making antibodies which can lyse tumor cells in the presence of human complement, it is possible to

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rapidly establish a panel of antibodies capable of reacting with and lysing a large variety of human tumors.

#### Therapeutic Uses of the Antibodies

5        When used in vivo for therapy, the antibodies of the subject invention are administered to the patient in therapeutically effective amounts (i.e. amounts that eliminate or reduce the patient's tumor burden). They will normally be administered parenterally, when possible, at the target cell site, or  
10       intravenously. The dose and dosage regimen will depend upon the nature of the cancer (primary or metastatic), its population, the site to which the antibodies are to be directed, the characteristics of the particular immunotoxin (when used), e.g., its therapeutic index, the patient, and the patient's history. The  
15       amount of antibody administered will typically be in the range of about 0.1 to about 10 mg/kg of patient weight.

For parenteral administration the antibodies will be formulated in a unit dosage injectable form (solution, suspension, emulsion) in association with a pharmaceutically acceptable  
20       parenteral vehicle. Such vehicles are inherently nontoxic, and non-therapeutic. Examples of such vehicles are water, saline, Ringer's solution, dextrose solution, and 5% human serum albumin. Nonaqueous vehicles such as fixed oils and ethyl oleate may also be  
25       used. Liposomes may be used as carriers. The vehicle may contain minor amounts of additives such as substances that enhance isotonicity and chemical stability, e.g., buffers and preservatives. The antibodies will typically be formulated in such vehicles at concentrations of about 1 mg/ml to 10 mg/ml.

30       The selection of an antibody subclass for therapy will depend upon the nature of the tumor antigen. For example, an IgM may be preferred in situations where the antigen is highly specific for the tumor target and rarely occurs on normal cells. However, where  
35       the tumor-associated antigen is also expressed in normal tissues,

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albeit at much lower levels, the IgG subclass may be preferred for the following reason: since the binding of at least two IgG molecules in close proximity is required to activate complement, less complement mediated damage may occur in the normal tissues which express smaller amounts of the antigen and, therefore, bind fewer IgG antibody molecules. Furthermore, IgG molecules by being smaller may be more able than IgM molecules to localize to tumor tissue.

There is evidence that complement activation in vivo leads to a variety of biological effects, including the induction of an inflammatory response and the activation of macrophages (Uanane and Benecerraf, Textbook of Immunology, 2nd Edition, Williams & Wilkins, p. 218 (1984)). Tumor cells are more sensitive to a cytolytic effect of activated macrophages than are normal cells, Fidler and Poste, Springer Semin. Immunopathol. 5, 161 (1982). The increased vasodilation accompanying inflammation may increase the ability of various anti-cancer agents, such as chemotherapeutic drugs, radiolabelled antibodies, etc., to localize in tumors. Therefore, antigen-antibody combinations of the type specified by this invention can be used therapeutically in many ways and may circumvent many of the problems normally caused by the heterogeneity of tumor cell populations. Additionally, purified antigens (Hakomori, Ann. Rev. Immunol. 2, 103 (1984)) or anti-idiotypic antibodies (Nepom et al., Proc. Natl. Acad. Sci. 81, 2864 (1985); Koprowski et al., Proc. Natl. Acad. Sci. 81, 216 (1984)) relating to such antigens could be used to induce an active immune response in human cancer patients. Such a response includes the formation of antibodies capable of activating human complement and mediating ADCC and by such mechanisms cause tumor destruction.

#### Immunoassays

Described herein are serological methods for determining the presence of HER2 p185. Essentially, the processes of this invention comprise incubating or otherwise exposing the sample to



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be tested to monoclonal antibodies and detecting the presence of a reaction product. Those skilled in the art will recognize that there are many variations of these basic procedures. These include, for example, RIA, ELISA, precipitation, agglutination, complement fixation and immuno-fluorescence. In the currently preferred procedures, the monoclonal antibodies are appropriately labeled.

The labels that are used in making labeled versions of the antibodies include moieties that may be detected directly, such as radiolabels and fluorochromes, as well as moieties, such as enzymes, that must be reacted or derivatized to be detected. The radiolabel can be detected by any of the currently available counting procedures. The preferred isotope labels are  $^{99}\text{Tc}$ ,  $^{14}\text{C}$ ,  $^{131}\text{I}$ ,  $^{125}\text{I}$ ,  $^3\text{H}$ ,  $^{32}\text{P}$  and  $^{35}\text{S}$ . The enzyme label can be detected by any of the currently utilized calorimetric, spectrophotometric, fluorospectro-photometric or gasometric techniques. The enzyme is combined with the antibody with bridging molecules such as carbodiimides, periodate, diisocyanates, glutaraldehyde and the like. Many enzymes which can be used in these procedures are known and can be utilized. Examples are peroxidase, alkaline phosphatase,  $\beta$ -glucuronidase,  $\beta$ -D-glucosidase,  $\beta$ -D-galactosidase, urease, glucose oxidase plus peroxidase, galactose oxidase plus peroxidase and acid phosphatase. Fluorescent materials which may be used include, for example, fluorescein and its derivatives, rhodamine and its derivatives, auramine, dansyl, umbelliferone, luciferia, 2,3-dihydrophthalazinediones, horseradish peroxidase, alkaline phosphatase, lysozyme, and glucose-6-phosphate dehydrogenase. The antibodies may be tagged with such labels by known methods. For instance, coupling agents such as aldehydes, carbodiimides, dimaleimide, imidates, succinimides, bis-diazotized benzidine and the like may be used to tag the antibodies with the above-described fluorescent, chemiluminescent, and enzyme labels. Various labeling techniques are described in Morrison, Methods in

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Enzymology 32b, 103 (1974), Syvanen et al., J. Biol. Chem. 284, 3762 (1973) and Bolton and Hunter, Biochem J. 133, 529 (1973).

5       The antibodies and labeled antibodies may be used in a variety  
of immunoimaging or immunoassay procedures to detect the presence  
of cancer in a patient or monitor the status of such cancer in a  
patient already diagnosed to have it. When used to monitor the  
status of a cancer, a quantitative immunoassay procedure must be  
used. If such monitoring assays are carried out periodically and  
10       the results compared, a determination may be made regarding whether  
the patient's tumor burden has increased or decreased. Common  
assay techniques that may be used include direct and indirect  
assays. If the sample includes cancer cells, the labeled antibody  
will bind to those cells. After washing the tissue or cells to  
15       remove unbound labeled antibody, the tissue sample is read for the  
presence of labeled immune complexes. In indirect assays the  
tissue or cell sample is incubated with unlabeled monoclonal  
antibody. The sample is then treated with a labeled antibody  
against the monoclonal antibody (e.g., a labeled antimurine  
20       antibody), washed, and read for the presence of ternary complexes.

For diagnostic use the antibodies will typically be  
distributed in kit form. These kits will typically comprise: the  
antibody in labeled or unlabeled form in suitable containers,  
25       reagents for the incubations for an indirect assay, and substrates  
or derivatizing agents depending on the nature of the label. HER2  
p185 controls and instructions may also be included.

30       The following examples are offered to more fully illustrate  
the invention, but are not to be construed as limiting the scope  
thereof.

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EXPERIMENTALAmplified Expression of p185<sup>HER2</sup> and Tyrosine Kinase Activity

5 A series of NIH 3T3 cell lines expressing various levels of p185 were constructed as disclosed in Hudziak *et al.*, Proc. Natl. Acad. Sci. (USA) 84, 7159 (1987). The parental cell line had a nontransformed, TNF- $\alpha$ -sensitive phenotype. The control cell line (NIH 3T3 neo/dhfr) was prepared by transfection with pCVN, an expression plasmid encoding neomycin resistance as a selectable marker, and dihydrofolate reductase (which encodes methotrexate resistance and which permits amplification of associated DNA sequences). pCVN-HER2 (which encodes, in addition, the entire 1255 amino acid p185 receptor-like tyrosine kinase under the transcriptional control of the RSV-LTR) was introduced into NIH 3T3 cells in a parallel transfection. Transfectants were selected by resistance to the aminoglycoside antibiotic G418. The pCVN-HER2 primary transfectants (HER2-3) do not have a transformed morphology and fail to grow in soft agar. Stepwise amplification of HER2 expression by selection in 200nM (HER2-3<sub>200</sub>), 400 nM (HER2-3<sub>400</sub>), and 800 nM (HER2-3<sub>800</sub>) methotrexate, however, results in transformation as judged by morphological criteria, the ability to grow in soft agar, and the ability to form tumors in nude mice.

25 The amplification of expression of p185 was documented by immunoprecipitation from cells that were metabolically labeled with <sup>35</sup>S-methionine. The tyrosine kinase activity associated with p185 in these cell lines was measured by autophosphorylation *in vitro*. For an autoradiograph of <sup>35</sup>S-methionine labeled p185, 200  $\mu$ Ci of <sup>35</sup>S-methionine (Amersham; 1132 Ci/mmol) was added to 1.5 ml of methionine-free labeling medium, containing 2% dialyzed fetal bovine serum. 1.0 x 10<sup>6</sup> cells of each type were counted by Coulter counter, plated in 60 mm culture dishes (Falcon), and allowed to adhere for 12 h. Following an 8 h labeling period the cells were lysed and the HER2-encoded p185 was analyzed. For an autoradiograph of self-phosphorylated HER2-receptor tyrosine kinase, the p185 was immunoprecipitated and the pellet was

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resuspended in 50  $\mu$ l of tyrosine kinase reaction buffer. The samples were incubated at 4°C for 20 min. The self-phosphorylated p185 from the various cell lines was then visualized by autoradiography following gel electrophoresis. The molecular weight markers used were myosin (200kD) and  $\beta$ -galactosidase (116kD). The results showed that expression of p185 and its associated tyrosine kinase increased in parallel during amplification. Quantitative densitometry of the *in vitro* autophosphorylation reactions showed that the tyrosine kinase activity increased at least 5 to 6-fold between HER2-3 and HER2-3200 and between HER2-3200 and HER2-3400, while only a small difference was observed between HER2-3400 and HER2-3800 (see the Tyrosine Kinase column of Table 1 below).

Relative amounts of tyrosine kinase present in each of the cell types of Table 1 were determined by taking ratios of the areas under the curves obtained by scanning autoradiograms (using an LKB2202 laser densitometer). The autoradiograms had been exposed for various times to allow for linearity in the determinations, and then normalized by comparison to the HER2 primary transfectant (HER2-3).

#### Resistance to TNF- $\alpha$

The cell lines described above were then tested for sensitivity to TNF- $\alpha$  and macrophage-induced cytotoxicity.

In Figure 1a, TNF- $\alpha$  resistance of the control cells and the HER2-transfected NIH 3T3 cells is shown. Cells were seeded into 96-well microtiter plates at a density of 5,000 cells/well in DMEM supplemented with 10% calf serum, 2mM L-glutamine, 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin. The cells were allowed to adhere for 4 hrs before the addition of a range of concentrations of TNF- $\alpha$ . Specific activity of the TNF- $\alpha$  (recombinant human TNF- $\alpha$ ) was  $5 \times 10^7$  U/mg as determined in an L-M cell cytotoxicity assay in the presence of actinomycin D. After incubation at 37°C for 72 hr,

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the monolayers were washed with PBS and stained with crystal violet dye for determination of relative cell viability. These measurements were repeated six times. Results from a representative experiment are shown in Figure 1a.

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In Figure 1b, macrophage-mediated cytotoxicity assays are shown. TNF- $\alpha$  resistant cells (neo/dhfr HTR) were derived by subculturing a clone of NIH 3T3 neo/dhfr in media containing 10,000 U/ml TNF- $\alpha$ . For macrophage cytotoxicity assays, NIH 3T3 neo/dhfr, HER2-3800 and neo/dhfr HTR cells were seeded into 96-well microtiter plates as in 1a above. Human macrophages were obtained as adherent cells from peripheral blood of healthy donors. Adherent cells were scraped and resuspended in media, activated for 4 hr. with 10  $\mu$ g/ml *E. coli*-derived lipopolysaccharide (LPS; Sigma) and 100 U/ml of recombinant human interferon-gamma (rHuIFN- $\gamma$ , Genentech, Inc.). The cell suspension was then centrifuged for 10 minutes at 1200 rpm and the resulting pellet was washed with media to remove the LPS and rHuIFN- $\gamma$ . The macrophages were resuspended in media, counted, and then added to the target cells to obtain the desired effector to target ratios. After a 72 hr incubation at 37°C, the monolayers were washed with media and  $^{51}\text{Cr}$  was added to each well for determination of viability by  $^{51}\text{Cr}$  uptake.

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Table 1

Correlation between HER2-associated tyrosine kinase levels and resistance to TNF- $\alpha$

5	Cell Type	Percent Viability	Tyrosine Kinase
10	1. NIH 3T3 neo/dhfr	3.6 $\pm$ 0.6	*
10	2. NIH 3T3 neo/dhfr <sub>400</sub>	8.3 $\pm$ 1.0	*
15	3. HER2-3	2.0 $\pm$ 0.4	1.0
15	4. HER2-3 <sub>200</sub>	27.5 $\pm$ 2.7	6.73
15	5. HER2-3 <sub>400</sub>	48.4 $\pm$ 1.4	32.48
15	6. HER2-3 <sub>800</sub>	58.7 $\pm$ 1.3	39.61
20	7. BT-20	1.6 $\pm$ 0.3	<0.1
20	8. MCF7	2.5 $\pm$ 0.3	0.26
20	9. MDA-MB-361	26.8 $\pm$ 6.6	10.65
20	10. MDA-MB-175-VII	31.2 $\pm$ 4.4	0.9
20	11. SK-BR-3	56.4 $\pm$ 5.5	31.0
20	12. MDA-MB-231	64.2 $\pm$ 9.3	<0.1
20	12. MDA-MB-231	64.2 $\pm$ 9.3	<0.1
20	12. MDA-MB-231	64.2 $\pm$ 9.3	<0.1
20	12. MDA-MB-231	64.2 $\pm$ 9.3	<0.1

\* not measured

25 Percent viability is given at  $1.0 \times 10^4$  cytotoxicity units per ml of TNF- $\alpha$ . The breast tumor cell lines were obtained from the ATCC and maintained in DMEM supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin.

30 As shown in Fig. 1a and Table 1, stepwise amplification of HER2 receptor expression resulted in a parallel induction of resistance to TNF- $\alpha$ . The primary transfectants (HER2-3), which do not have a transformed phenotype, demonstrated little increased resistance. However, the transformed lines HER2-3<sub>200</sub>, HER2-3<sub>400</sub> and HER2-3<sub>800</sub> do show a stepwise loss in sensitivity to TNF- $\alpha$ -mediated cytotoxicity as compared to NIH 3T3 neo/dhfr (Fig. 1a and Table 1), although the MDA-MB-175-VII cells had elevated p185 expression compared to the TNF- $\alpha$  sensitive BT20 and MCF7 cell lines. In correlation with the levels of p185 expression (Table 1), the difference in sensitivity of HER2-3<sub>200</sub> and HER2-3<sub>400</sub> (27.5%

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vs. 48.4% viability at  $1 \times 10^4$  U/ml TNF- $\alpha$ ) is greater than the difference between HER2-3400 and HER2-3800 (48.4% vs. 58.7% viability, see Fig. 1a and Table 1). A similar result was obtained when NIH 3T3 neo/dhfr and HER2-3800 were compared for sensitivity to activated macrophages (Fig. 1b). These data suggest that amplification of the expression of HER2 induces resistance to TNF- $\alpha$ , and also show that this correlates with resistance to an important component of the early host defense mechanism, the activated macrophage. Amplification of the control plasmid (pCVN) in the cell line NIH 3T3 neo/dhfr<sub>400</sub> did not induce increased resistance to TNF- $\alpha$  (Table 1). This demonstrates that neither gene transfection or gene amplification, per se, has any effect on the sensitivity of cells to TNF- $\alpha$ .

The observation that NIH 3T3 cell lines expressing high levels of p185 were resistant to cytotoxicity induced by TNF- $\alpha$  or macrophages suggested that this may be one mechanism leading to tumor development. To test this possibility six breast tumor cell lines were screened for amplification of HER2 and sensitivity to TNF- $\alpha$ -mediated cytotoxicity. The results (Table 1) demonstrated that sensitivity to growth inhibition by TNF- $\alpha$  is inversely correlated with the expression of HER2-associated tyrosine kinase measured in vitro autophosphorylation assay for BT-20, MCF7, MDA-MB-361 and SK-BR-3. Two of the TNF- $\alpha$ -resistant breast tumor cell lines (MDA-MB-175-VII and MDA-MB-231), however, had no demonstrable amplified expression of HER2 as compared to the HER2-3 control (Table 1), although the MDA-MB-175-VII cells had elevated p185 expression compared to the TNF- $\alpha$  sensitive BT20 and MCF7 cell lines. These results are consistent with previous reports of the frequency of HER2 amplification in primary breast tumors and tumor-derived cell lines, and suggest the existence of other cellular mechanisms which may lead to TNF- $\alpha$  resistance.

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Experiments also showed that overexpression of the EGF receptor, and cellular transformation by the src oncogene, correlates with resistance to TNF- $\alpha$ .

5     TNF- $\alpha$  Receptor Binding

10     In order to investigate whether the TNF- $\alpha$  receptor was altered in HER2-3800, as opposed to NIH 3T3 neo/dhfr, the binding of  $^{125}\text{I}$ -labeled TNF- $\alpha$  was compared between these cell lines. Figure 2 shows a TNF- $\alpha$  receptor binding analysis. Displacement curves show  
15     binding of  $^{125}\text{I}$ -TNF- $\alpha$  to NIH 3T3 neo/dhfr and HER2-3800. Competition binding assays were performed. Briefly, a suspension of  $2.0 \times 10^6$  cells were incubated in a final volume of 0.5 ml of RPMI-1640 medium containing 10% fetal bovine serum. Binding of  $^{125}\text{I}$ -TNF- $\alpha$  ( $0.2 \times 10^6$  cpm) to the cells was determined in the  
20     presence or absence of varying concentrations of unlabeled TNF- $\alpha$  at  $4^\circ\text{C}$  under saturation equilibrium conditions. Each data point represents the mean of triplicate determinations. After incubation overnight, cells were washed twice with incubation buffer and cell bound radioactivity was determined. Non-specific binding was <10% of total binding.

25     The results showed a 2-3 fold increase in total specific binding for HER2-3800 as compared to NIH 3T3 neo/dhfr (Fig. 2). In addition, the displacement curve for binding on HER2-3800 is also shifted toward lower affinity binding as compared to NIH 3T3 neo/dhfr (Fig. 2).

Production of Anti-HER2 Monoclonal Antibodies

30     Five female Balb/c mice were immunized with HER2 amplified NIH 3T3 transformed cells over a period of 22 weeks. The first four injections each had approximately  $10^7$  cells/mouse. They were administered intraperitoneally in half a milliliter of PBS on weeks 0, 2, 5, 7. Injections five and six were with a wheat germ agglutinin partially purified membrane preparation which had a  
35     whole protein concentration of about 700  $\mu\text{g/ml}$ . A 100  $\mu\text{l}$ /injection



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was administered to each mouse intraperitoneally on weeks 9 and 13. The last injection was also with the purified material but was administered three days prior to the date of fusion intravenously.

5           Bleeds from the mice were tested at various times in a radioimmunoprecipitation using whole cell lysates. The three mice with the highest antibody titers were sacrificed and spleens were fused with the mouse myeloma cell line X63-Ag8.653 using the general procedure of Mishell & Shiigi, Selected Methods in Cellular Immunology, W.H. Freeman & Co., San Francisco, p. 357-363 (1980) with the following exceptions. Cells were plated at a density of approximately  $2 \times 10^5$  cells/well into ten 96 well microtiter plates. Hybrids were selected using hypoxanthine-azoserine rather than hypoxanthine-aminopterin-thymidine (HAT).

15           Hybridoma supernatants were tested for presence of antibodies specific for HER2 receptor by ELISA and radioimmunoprecipitation.

20           For the ELISA,  $3.5 \mu\text{g/ml}$  of the HER2 receptor (purified on the wheat germ agglutinin column) in PBS was adsorbed to immulon II microtiter plates overnight at  $4^\circ\text{C}$  or for 2 hours at room temperature. Plates were then washed with phosphate buffered saline with .05% Tween 20 (PBS-TW20) to remove unbound antigen. Remaining binding sites were then blocked with  $200 \mu\text{l}$  per well of 25   1% bovine serum albumin (BSA) in PBS-TW20 and incubated 1 hour at room temperature. Plates were washed as above and  $100 \mu\text{l}$  of hybridoma supernatant was added to each well and incubated for 1 hour at room temperature. Plates were washed again and  $100 \mu\text{l}$  per well of an appropriate dilution of goat anti-mouse immunoglobulin 30   coupled to horseradish peroxidase was added. The plates were incubated again for 1 hour at room temperature and then washed as above. O-phenylene diamine was added as substrate, incubated for 15-20 minutes at room temperature and then the reaction was stopped with  $2.5 \text{ M H}_2\text{SO}_4$ . The absorbance of each well was then read at 492 35   nm.

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For the radioimmunoprecipitation, first the wheat germ purified HER2 receptor preparation was autophosphorylated in the following manner: a kinase solution with the following final concentrations was made: .18 mCi/ml  $\gamma$ P<sup>32</sup>-ATP (Amersham), .4mM MgCl<sub>2</sub>, .2mM MnCl<sub>2</sub>, 10 $\mu$ M ATP, 35  $\mu$ g/ml total protein concentration of partially purified HER2 all diluted in 20mM Hepes, 0.1% triton 10% glycerol buffer (HTG). This reaction was incubated for 30 minutes at room temperature. 50  $\mu$ l hybridoma supernatant was then added to 50  $\mu$ l of the kinase reaction and incubated 1 hour at room temperature. 50  $\mu$ l of goat anti-mouse IgG precoated protein-A sepharose CL4B, at a sepharose concentration of 80 mg/ml, was added to each sample and incubated 1 hour at room temperature. The resulting immunocomplexes were then washed by centrifugation twice with HTG buffer and finally with .2% deoxycholate .2% Tween 20 in PBS, in a microfuge and aspirated between washes. Reducing sample buffer was added to each sample and samples were heated at 95°C for 2-5 minutes, insoluble material was removed by centrifugation and the reduced immunocomplex was loaded onto a 7.5% polyacrylamide gel containing SDS. The gel was run at 30 amp constant current and an autoradiograph was obtained from the finished gel.

Approximately 5% of the total well supernatants reacted with the HER2 receptor in the ELISA and/or radioimmunoprecipitation. From this initial 5% (about 100), some hybrids produced low affinity antibodies and others suffered from instability and stopped secreting antibodies leaving a total of 10 high affinity stable HER2 specific antibody producing cell lines. These were expanded and cloned by limiting dilution (Oi, V.T. and Herzenberg, L.A., "Immunoglobulin Producing Hybrid Cell Lines" in Selected Methods in Cellular Immunology, p. 351-372 Mishell, B.B. and Shiigi, S.M. (eds.), W.H. Freeman and Co. (1980)). Large quantities of specific monoclonal antibodies were produced by injection of cloned hybridoma cells in pristaned primed mice to

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produce ascitic tumors. Ascites were then collected and purified over a protein-A sepharose column.

#### Screening of Antibodies

5       The 10 high affinity monoclonal antibodies were then screened in a number of assays for anti-transformation or anti-tumor cell activity. Monoclonal antibodies were selected on the basis of growth inhibiting activity against the human tumor line SK BR3 which is derived from a breast tumor and contains an amplified HER2 gene and overexpresses the HER2 p185 tyrosine kinase. The initial screen used conditioned medium (medium in which the cells were grown for several days containing any secreted products of the cells including antibodies produced by the cells) from the hybridoma cell lines.

15       SK BR3 cells were plated at 20,000 cells/35 mm dish. Either conditioned medium from the hybridoma parent line (producing everything but anti-HER2 monoclonals) as a control, or the anti-HER2 monoclonals were added. After 6 days, the total number of SK BR3 cells were counted using an electronic Coulter cell counter. Cells were grown in a 1:1 mixture of F-12 and DMEM supplemented with 10% fetal bovine serum, glutamine, and penicillin/streptomycin. The volume per plate was 2 mls/35mm dish. 0.2 mls of myeloma conditioned medium was added per 35mm dish. Each control or anti-HER2 MAb was assayed in duplicate and the two counts averaged.

25       The result of the survey is shown in Figure 3. Monoclonal antibody 4D5 markedly inhibited the growth of the breast tumor line SK BR3. Other anti-HER2 antibodies inhibited growth to a significant but lesser extent (egs., MAbs 3E8 and 3H4). Still other anti-HER2 antibodies (not shown) did not inhibit growth.

30       A repeat experiment using purified antibody rather than hybridoma conditioned medium confirmed the results of Figure 3. Figure 4 is a dose response curve comparing the effect of an

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irrelevant monoclonal antibody (anti-HBV) and monoclonal antibody 4D5 (anti-HER2) on the growth of the SK BR3 cell line in 10% fetal bovine serum.

5     Down Regulation of the HER2 Receptor

10     The SK BR3 cells were pulse labeled with 100 $\mu$ Ci  $^{35}$ S-methionine for 12 hours in methionine-free medium. Then either an irrelevant (anti-Hepatitis B surface antigen) or anti-HER2 MAb (4D5) was added to the cells at 5  $\mu$ g/ml. After 11 hours, the cells were lysed and  
15     HER2 p185 immunoprecipitates and the proteins were analyzed by a 7.5% acrylamide gel followed by autoradiography. The SDS-PAGE gel of the  $^{35}$ S-methionine labeled HER2 p185 from SK BR3 cells demonstrated that the HER2 levels are downregulated by MAb 4D5.

15     Treatment of Breast Tumor Cells with Monoclonal Antibodies and TNF- $\alpha$

20     SK-BR-3 breast tumor cells were seeded at a density of  $4 \times 10^4$  cells per well in 96-well microtiter plates and allowed to adhere for 2 hours. The cells were then treated with different concentrations of anti-HER2 monoclonal antibody (MAb) 4D5 or  
25     irrelevant isotype matched (anti-rHuIFN- $\gamma$  MAb) at 0.05, 0.5 or 5.0  $\mu$ g/ml for 4 hours prior to the addition of 100, 1,000 or 10,000 U/ml rHuTNF- $\alpha$ . After a 72 hour incubation, the cell monolayers were stained with crystal violet dye for determination of relative  
30     percent viability (RPV) compared to control (untreated) cells. Each treatment group consisted of 6 replicates. The results are shown in Figures 5 and 6. These Figures show that incubation of cells overexpressing HER2 receptor with antibodies directed to the extracellular domain of the receptor induce sensitivity to the cytotoxic effects of TNF- $\alpha$ . Equivalent treatment of breast tumor cells MDA-MB-175 VII gave similar results (see Figure 7). Treatment of human fetal lung fibroblasts (WI-38) with MAb resulted in no growth inhibition or induction of sensitivity to TNF- $\alpha$  as expected.

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Treatment of NIH 3T3 Cells Overexpressing HER2 p185 with Monoclonal Antibodies and TNF- $\alpha$

NIH 3T3 HER2-3400 cells were treated with different concentrations of anti-HER2 MAb as in the above described treatment of SK-BR3 cells. The results for MAb 4D5 are shown in Figure 8. The results indicate that cells other than of breast tumor cell lines which overexpress the HER2 receptor are growth inhibited by antibodies to the HER2 receptor, and sensitivity to TNF- $\alpha$  is induced in the presence of these antibodies.

In Vivo Treatment of NIH 3T3 Cells Overexpressing HER2 with Anti-HER2 IgG2A Monoclonal Antibodies

NIH 3T3 cells transfected with either a HER2 expression plasmid (NIH 3T3<sub>400</sub>) or the neo-DHFR vector were injected into nu/nu (athymic) mice subcutaneously at a dose of  $10^6$  cells in 0.1 ml of phosphate-buffered saline. On days 0, 1, 5 and every 4 days thereafter, 100  $\mu$ g (0.1 ml in PBS) of either an irrelevant or anti-HER2 monoclonal antibody of the IgG2A subclass was injected intraperitoneally. Tumor occurrence and size were monitored for the 1 month period of treatment.

Table 2

<u>Group #</u>	<u>Cell Line</u>	<u>Treatment</u>	<u># Tumors/ # Animals</u>	<u>Tumor Size of Survivors: Length x Width Average in mm<sup>2</sup> at 31 Days</u>
1	HER2 (3T3 <sub>400</sub> )	Irrelevant MAb (anti-Hepatitis B Virus)	6/6	401
2	HER2 (3T3 <sub>400</sub> )	2H11 anti-HER2	2/6	139
3	HER2 (3T3 <sub>400</sub> )	3E8 anti-HER2	0/6	0
4	neo/DHFR	None	0/6	0

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Table 2 shows that the 2H11 MAb has some anti-tumor activity (MAb 2H11 has very slight growth inhibiting properties when screened against tumor line SK BR3) and the 3E8 MAb gives 100% tumor growth inhibition during the course of the experiment.

5

While the invention has been described in what is considered to be its preferred embodiments, it is not to be limited to the disclosed embodiments, but on the contrary, is intended to cover various modifications and equivalents included within the spirit and scope of the appended claims, which scope is to be accorded the broadest interpretation so as to encompass all such modifications and equivalents.

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What is claimed is:

1. A monoclonal antibody specifically binding the extracellular domain of the HER2 receptor.
- 5 2. A monoclonal antibody as in claim 1 which is capable of inhibiting the HER2 receptor function.
3. A monoclonal antibody as in claim 1 which is capable of inhibiting serum activation of HER2 receptor function.
- 10 4. A monoclonal antibody as in claim 1 which is a murine monoclonal antibody.
5. A monoclonal antibody as in claim 1 which is a murine-human hybrid antibody.
- 15 6. A monoclonal antibody as in claim 1 wherein said antibody specifically blocks the ligand binding site of the HER2 receptor.
- 20 7. A monoclonal antibody as in claim 1 which down regulates the HER2 receptor.
8. A monoclonal antibody as in claim 1 wherein said antibody is capable of activating complement.
- 25 9. A monoclonal antibody as in claim 1, wherein said antibody is capable of mediating antibody dependent cellular cytotoxicity.
10. An immunotoxin which is a conjugate of a cytotoxic moiety and the antibody of claim 1.
- 30 11. A hybridoma producing the monoclonal antibody of claim 1.

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12. An assay for detecting a tumor comprising the steps of:  
    exposing cells to antibodies specifically binding the  
    extracellular domain of the HER2 receptor, and  
    determining the extent of binding of said antibodies to  
5 said cells.
13. An assay as in claim 12 wherein said antibodies are monoclonal  
antibodies.
- 10 14. An assay as in claim 12 wherein the assay is an ELISA assay.
- 15 15. An assay as in claim 12 wherein said cells remain within the  
body of a mammal, said antibodies are tagged with a radioactive  
isotope and administered to the mammal, and the extent of binding  
of said antibodies to said cells is observed by external scanning  
for radioactivity.
- 20 16. A method of inhibiting the growth of tumor cells comprising:  
    administering to a patient a therapeutically effective  
amount of antibodies capable of inhibiting the HER2 receptor  
function.
- 25 17. A method as in claim 16 wherein said antibodies specifically  
blocks the ligand binding site of the HER2 receptor.
- 30 18. A method as in claim 16 wherein said antibodies are conjugated  
to a cytotoxic moiety.
19. A method as in claim 16 wherein said antibodies are capable of  
activating complement.
20. A method as in claim 16 wherein said antibodies are capable of  
mediating antibody dependent cellular cytotoxicity.



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21. A method as in claim 16 wherein said antibodies are monoclonal antibodies.
- 5 22. A method as in claim 16 wherein the tumor cells comprise a carcinoma selected from human breast, renal, gastric and salivary gland carcinomas, or other tumor cell types expressing the HER2 receptor.
- 10 23. A method of treating tumor cells comprising the steps of:  
administering to a patient a therapeutically effective amount of antibodies capable of inhibiting growth factor receptor function; and  
administering to a patient a therapeutically effective amount of a cytotoxic factor.
- 15 24. A method as in claim 23 wherein said cytotoxic factor is selected from the group consisting of TNF- $\alpha$ , TNF- $\beta$ , IL-1, IFN- $\gamma$  and IL-2.
- 20 25. A method as in claim 23 wherein said cytotoxic factor is TNF- $\alpha$ .
26. A method as in claim 23 wherein said antibodies interrupt an autocrine growth cycle.
- 25 27. A method as in claim 23 wherein said antibodies specifically bind a growth factor receptor.
- 30 28. A method as in claim 27 wherein the growth factor receptor is selected from the group consisting of the EGF receptor and the HER2 receptor.
29. A method as in claim 23 wherein said antibodies specifically bind a growth factor.

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30. A method as in claim 29 wherein said growth factor is selected from the group consisting of EGF, TGF- $\alpha$  and TGF- $\beta$ .
- 5 31. A method as in claim 23 wherein said antibodies are monoclonal antibodies.
32. A method as in claim 23 wherein said antibodies are conjugated to a cytotoxic moiety.
- 10 33. A method as in claim 23 wherein said antibodies are capable of activating complement.
34. A method as in claim 23 wherein said antibodies are capable of mediating antibody dependent cellular cytotoxicity.
- 15 35. A method as in claim 23 wherein the tumor cells comprise a carcinoma selected from human breast, renal, gastric and salivary gland carcinomas.
- 20 36. An assay for receptors and other proteins having increased tyrosine kinase activity comprising the steps of:
- (a) exposing cells suspected to be TNF- $\alpha$  sensitive to TNF- $\alpha$ ;
  - (b) isolating those cells which are TNF- $\alpha$  resistant;
  - (c) screening the isolated cells for increased tyrosine kinase activity; and
  - 25 (d) isolating receptors and other proteins having increased tyrosine kinase activity.
- 30 37. A composition suitable for administration to a patient having a growth factor receptor dependent tumor comprising (a) antibodies capable of inhibiting growth factor receptor function, and (b) a cytotoxic factor.

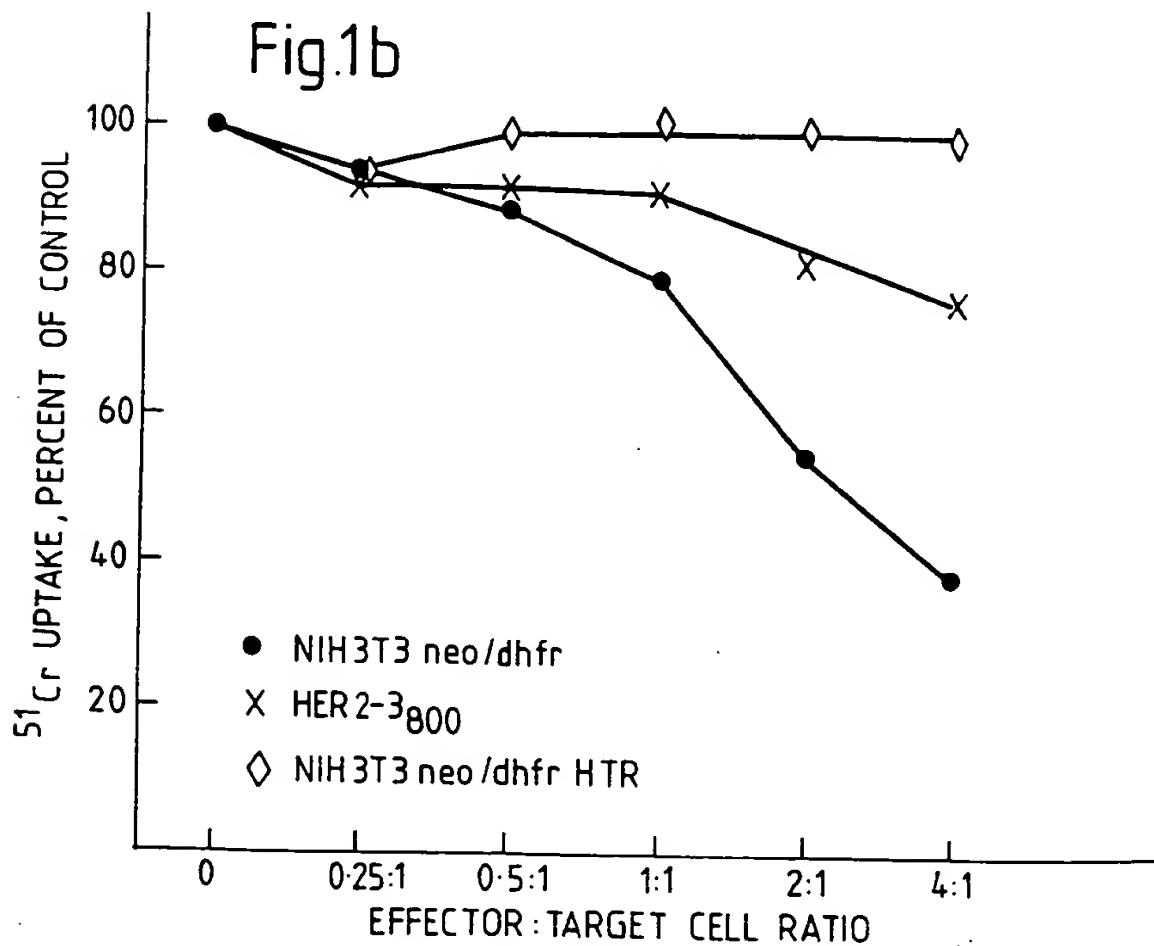
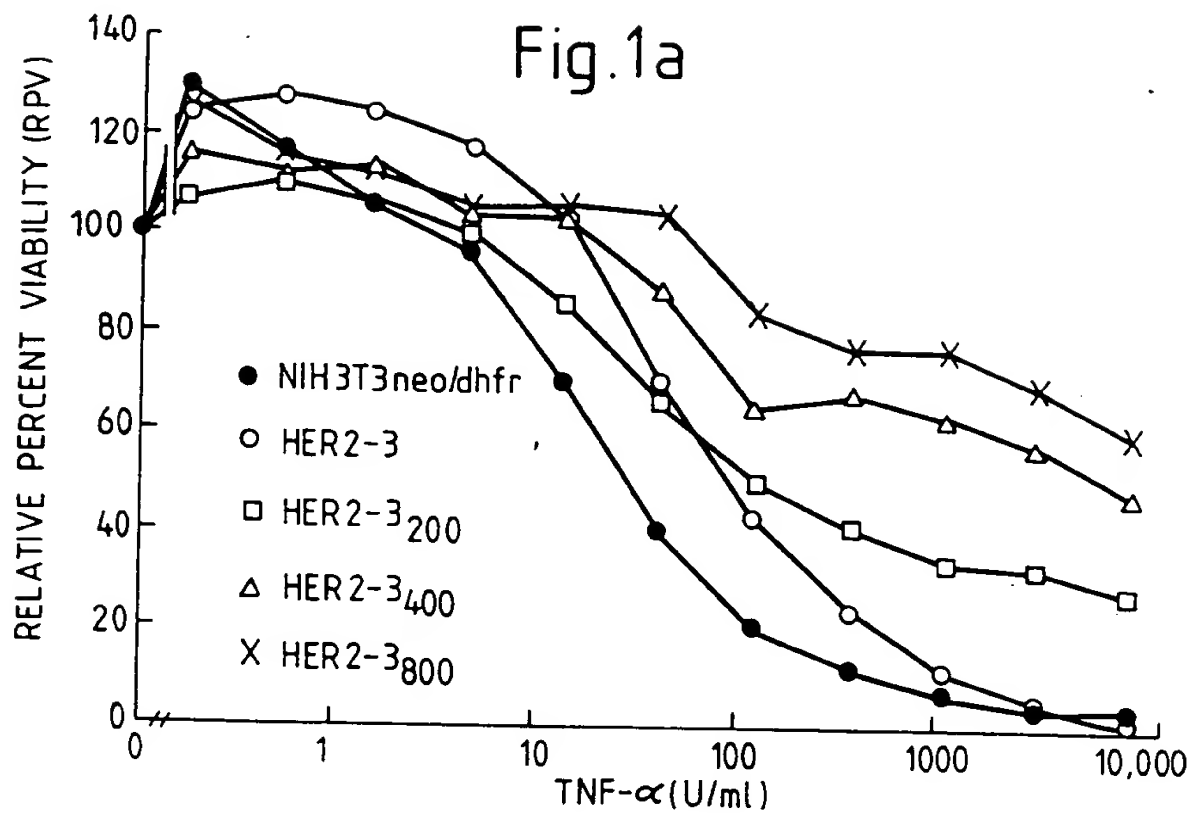
-41-

38. A composition as in claim 37 wherein the cytotoxic factor is selected from the group consisting of TNF- $\alpha$ , TNF- $\beta$ , IL-1, IFN- $\gamma$  and IL-2.

5 39. An immunotoxin as in Claim 10 wherein the cytotoxic moiety is ricin A chain.

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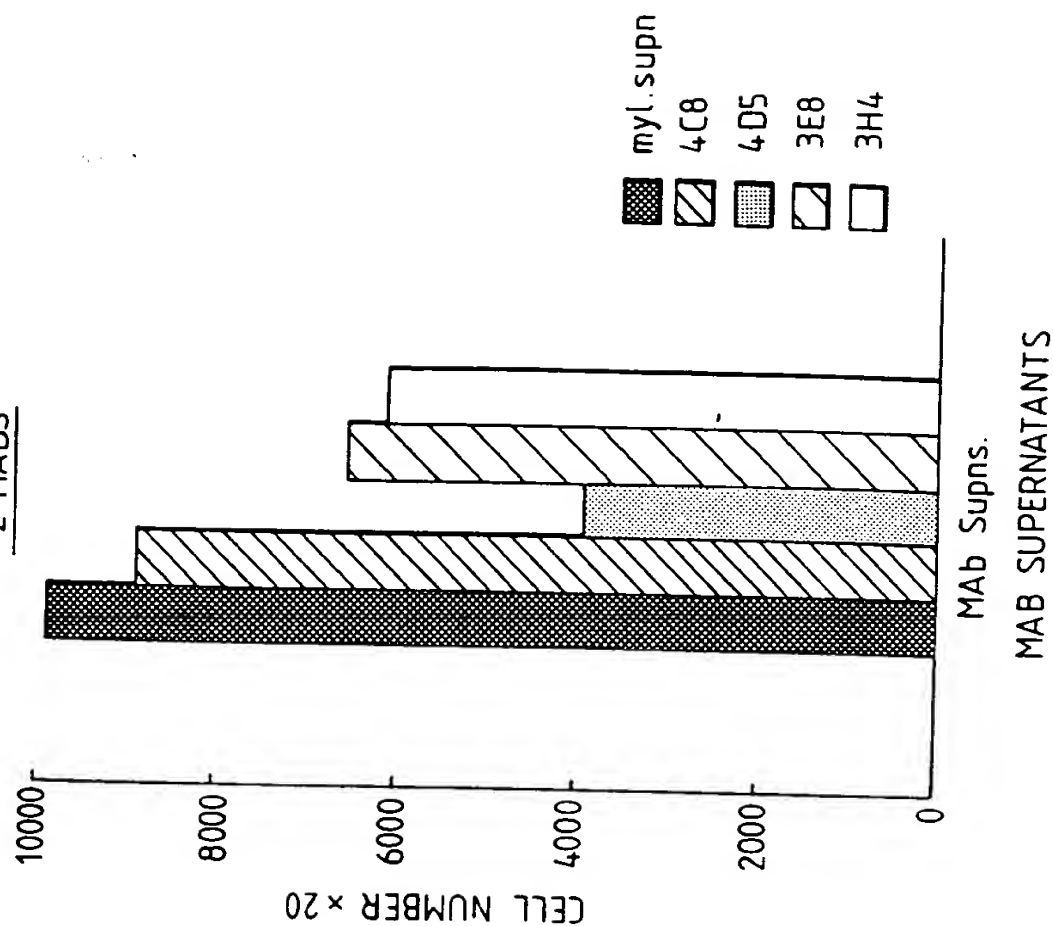
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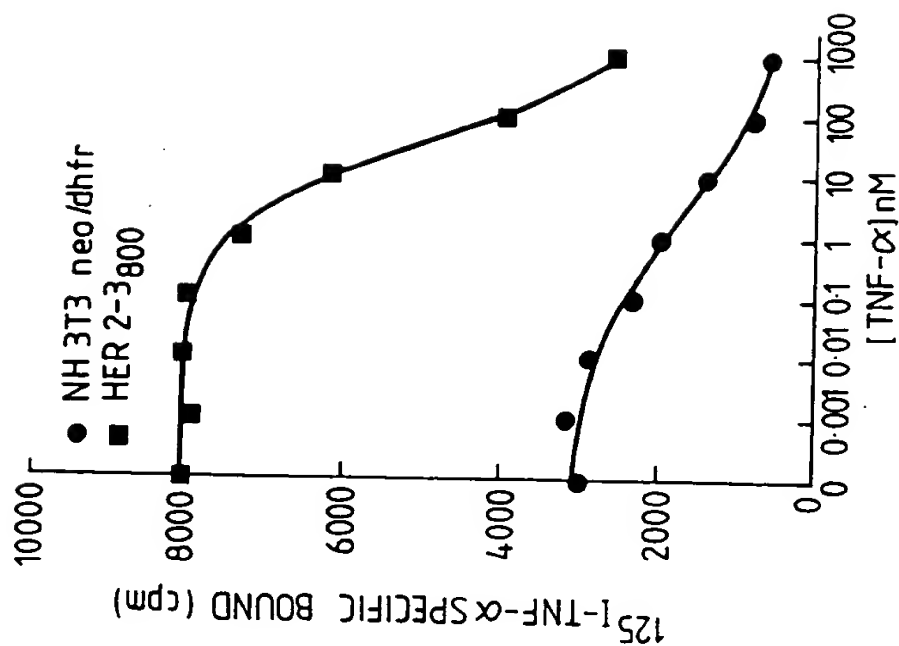
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**Fig. 3**  
INHIBITION OF SKBR 3 GROWTH BY ANTI-HER  
-2 MABS



**Fig. 2**

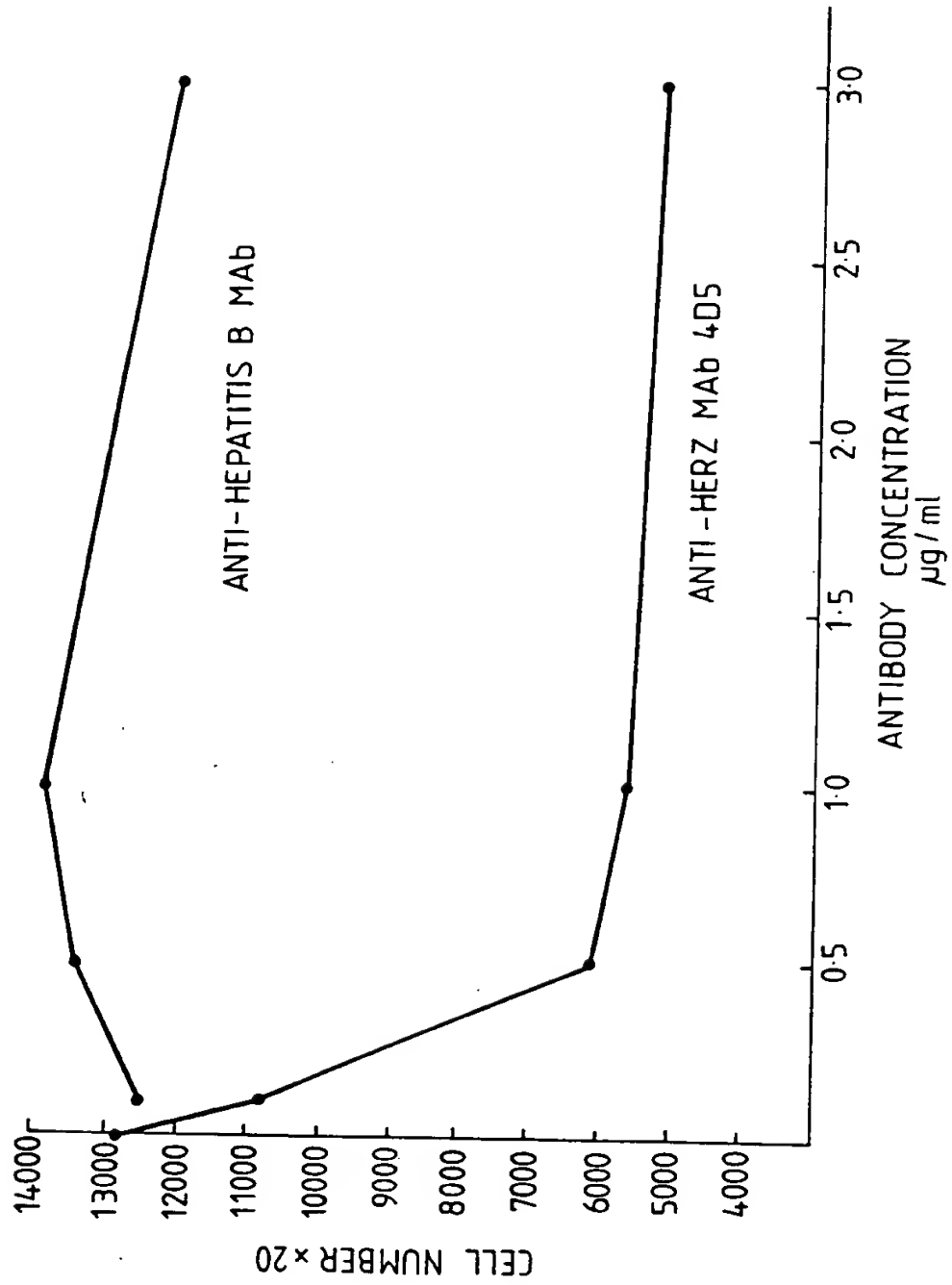


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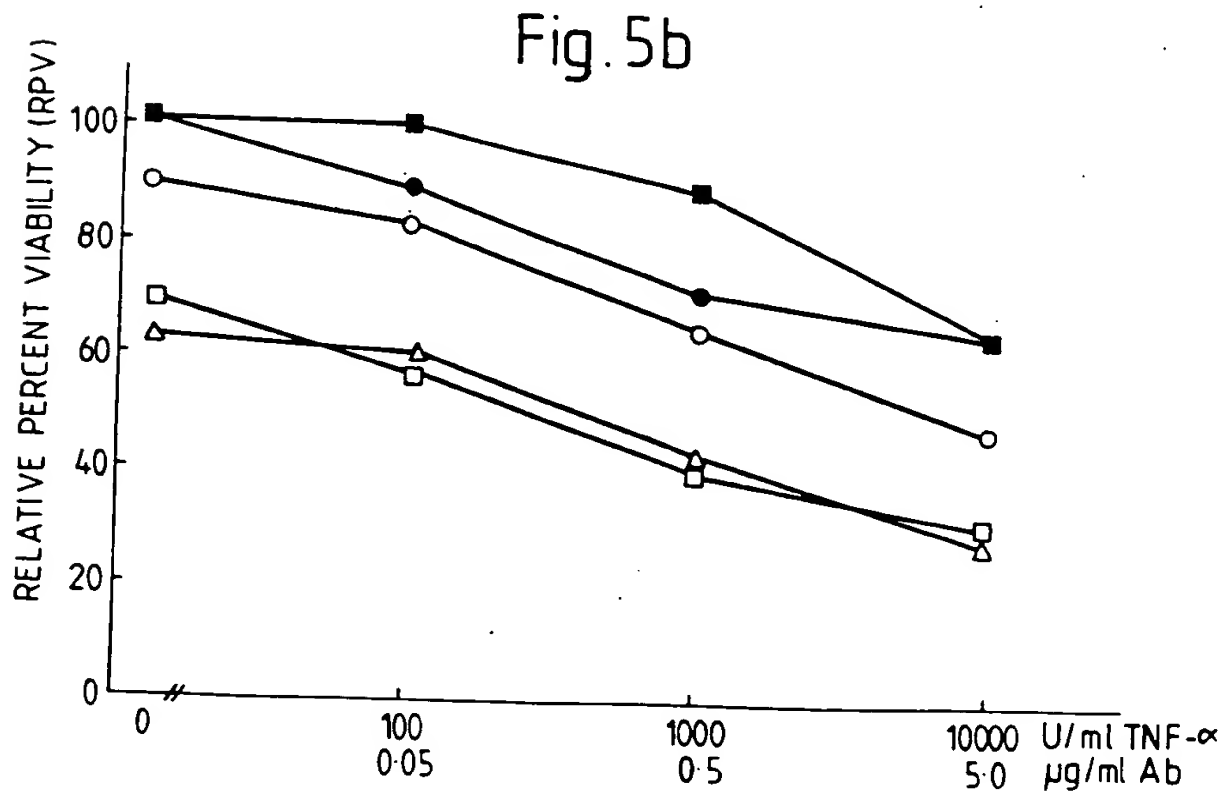
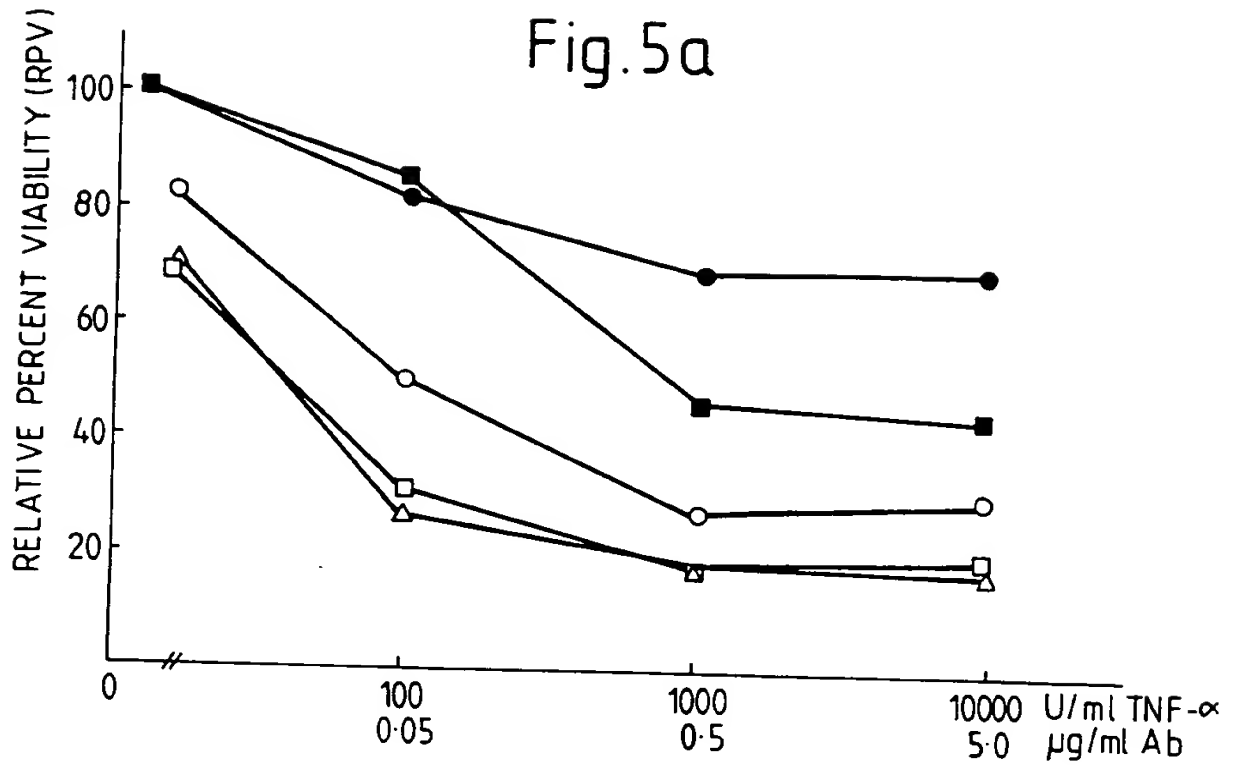
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Fig. 4



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Fig. 6a

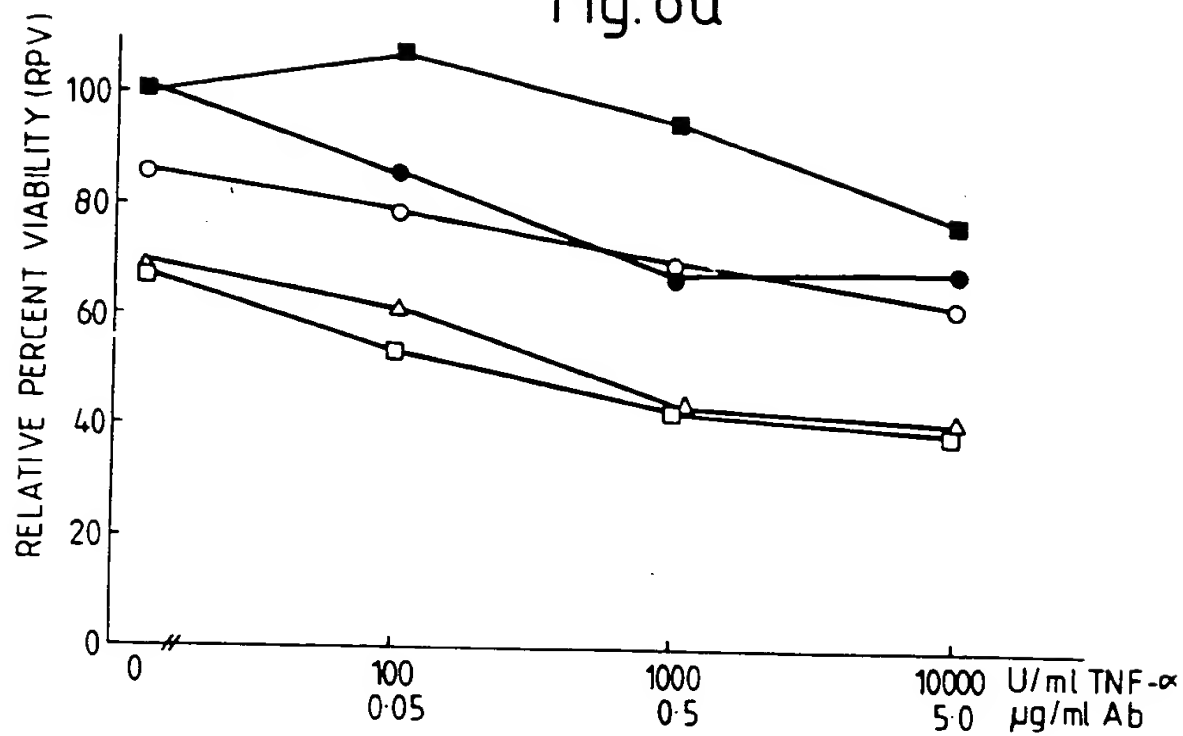
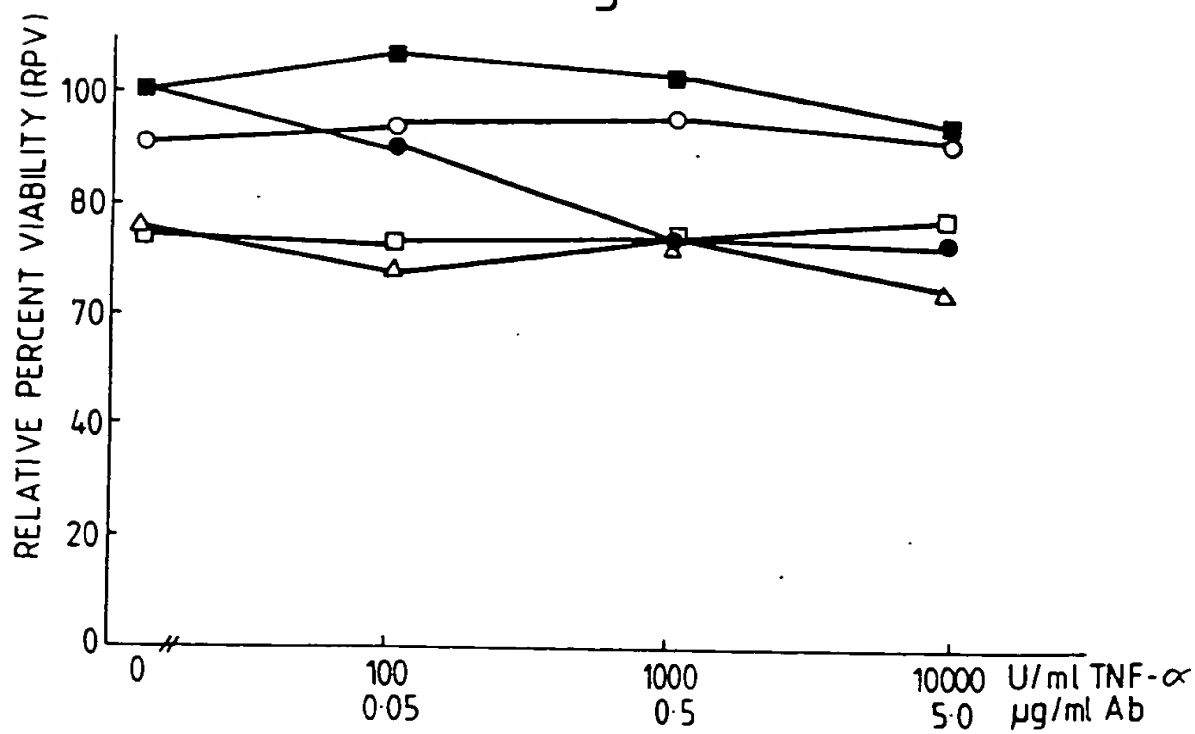
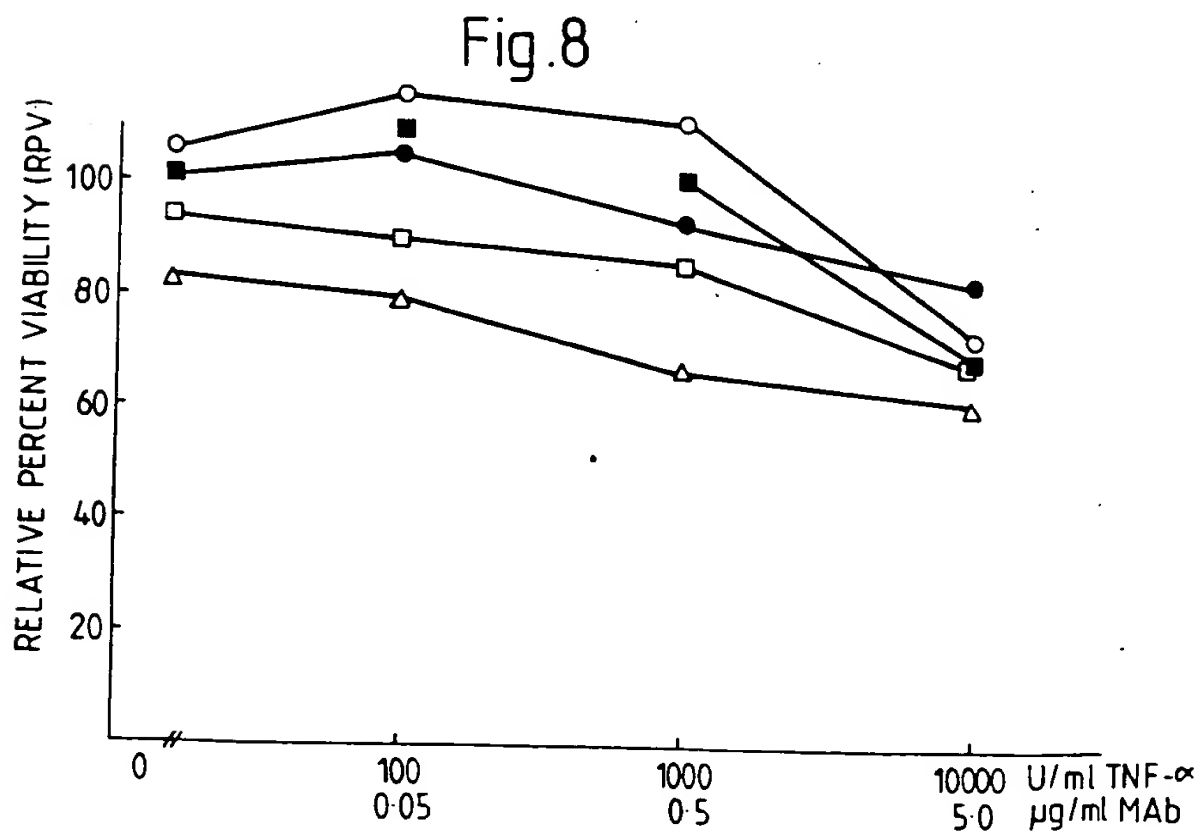
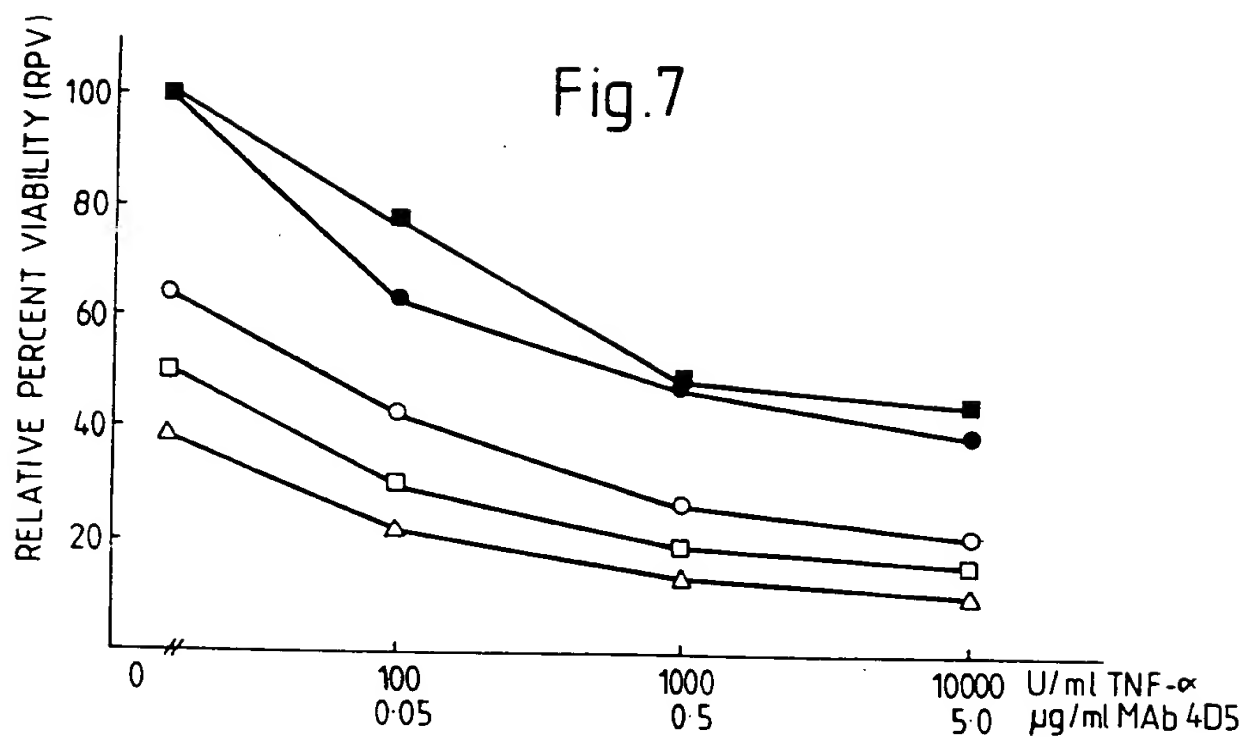


Fig. 6b



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# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 89/00051

## I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) \*

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC<sup>4</sup>: C 12 P 21/00; C 12 N 15/00; C 12 N 5/00; G 01 N 33/574;  
A 61 K 39/395

## II. FIELDS SEARCHED

Minimum Documentation Searched ?	
Classification System	Classification Symbols
IPC <sup>4</sup>	A 61 K; C 12 N

Documentation Searched other than Minimum Documentation  
to the extent that such Documents are included in the Fields Searched \*

## III. DOCUMENTS CONSIDERED TO BE RELEVANT \*

Category *	Citation of Document, ** with indication, where appropriate, of the relevant passages **	Relevant to Claim No. **
Y	WO, A, 85/03357 (ICRF PATENTS LTD; YEDA RESEARCH & DEVELOPMENT CO. LTD; GENENTECH INC.) 1 August 1985 see page 36, line 15 - page 40, line 3	12-15
Y	J. Natl. Cancer Instit., vol. 79, no. 3, September 1987, R.E. Sobol et al.: "Epidermal growth factor receptor expression in human lung carcinomas defined by a mono- clonal antibody", pages 403-407 see page 403, abstract	12-15
Y	Cancer Res., vol. 47, no. 14, July 1987, U. Rodeck et al.: "Tumor growth modulation by a monoclonal antibody to the epidermal growth factor receptor: immunologically mediated and effector cell-independent effects", pages 3692-3696	1-9

\* Special categories of cited documents: \*\*

"A" document defining the general state of the art which is not  
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filing date

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other means

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or priority date and not in conflict with the application but  
cited to understand the principle or theory underlying the  
invention

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cannot be considered novel or cannot be considered to  
involve an inventive step

"Y" document of particular relevance; the claimed invention  
cannot be considered to involve an inventive step when the  
document is combined with one or more other such docu-  
ments, such combination being obvious to a person skilled  
in the art.

"Z" document member of the same patent family

## IV. CERTIFICATION

Date of the Actual Completion of the International Search

2nd May 1989

Date of Mailing of this International Search Report

16.06.89

International Searching Authority

EUROPEAN PATENT OFFICE

Signature of Authorizing Officer

P.C.G. VAN DER PUTTEN

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
	see page 3695 --	
Y	Cancer Res., vol. 46, no. 11, November 1986, H. Masui et al.: "Mechanism of anti-tumor activity in mice for anti-epidermal growth factor receptor monoclonal antibodies with different isotypes", pages 5592-5598 see page 5592, abstract --	1-9
Y	Proc. Natl. Acad. Sci., USA, vol. 83, December 1986, J.A. Drebin et al.: "Inhibition of tumor growth by a monoclonal antibody reactive with an oncogene-encoded tumor antigen", pages 9129-9133 see page 9129, abstract cited in the application --	1-9
Y	Journal of Cellular Biochemistry, Growth Regulation of Cancer, vol. 35, no. 4, 1987, Alan R. Liss, Inc., U. Rodeck et al.: "Interactions between growth factor receptors and corresponding monoclonal antibodies in human tumors", pages 315:JCB-320:JCB, 65:GRC-70:GRC see page 65:GRC --	1-9
Y	Cell, vol. 41, July 1985, MIT, J.A. Drebin et al.: "Down-modulation of an oncogene protein product and reversion of the transformed phenotype by monoclonal antibodies", pages 695-706 see page 695, abstract cited in the application -----	1-9

**ANNEX TO THE INTERNATIONAL SEARCH REPORT  
ON INTERNATIONAL PATENT APPLICATION NO.**

US 8900051

SA 26543

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 09/06/89  
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A- 8503357	01-08-85	AU-A- 3934085	09-08-85
		EP-A- 0171407	19-02-86
		JP-T- 61501168	12-06-86
-----			

**EPO FORM 10479**

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

## FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☒ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE <sup>1</sup>

This International search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☒ Claim numbers \*..... because they relate to subject matter not required to be searched by this Authority, namely:  
 \* 16-35, see PCT-rule 39.1(IV); methods for treatment of the human or animal body by surgery or therapy, as well as diagnostic methods.

2. ☐ Claim numbers..... because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim numbers..... because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING <sup>2</sup>

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

## Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

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